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Multiplex Polymerase Chain Reaction for Subgenus-Specific Detection of Human Adenoviruses in Clinical Samples

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The polymerase chain reaction (PCR) has been used previously for the detection and typing of adenoviruses directly in clinical samples. Since under clinical conditions subgenus-specific identification is often sufficient, we extended the genus- and type-specific PCR by a subgenus-specific PCR. By sequencing several loop l_4 gene regions of the hexon (major adenovirus coat protein) and comparing them to published sequences, subgenus-specific sequences were identified in this region. By using primers targeted to this region and to a conserved hexon gene region, a multiplex, nonnested PCR for the detection and subgenus-specific identification of adenoviruses could be established. The six subgenus-specific amplimers are distinguishable by agarose gel electrophoresis, and subsequent restriction analysis is not necessary. The specificity of the subgenus-specific primer pairs was tested on 23 adenovirus prototypes, representing all six subgenera, on 9 subgenus B and D intermediate strains, and on 16 subgenus C genome types. Furthermore, multiplex, subgenus-specific PCR was performed directly with 100 clinical specimens, including stool samples, ocular swabs, and throat swabs. Adenoviruses of all subgenera could be detected. Especially for clinical application, the rapid, one-step differentiation between subgenus D adenoviruses, causing the severe and highly contagious epidemic keratoconjunctivitis, and subgenus B and E adenoviruses, causing relative harmless ocular infections, is of great importance. The subgenus-specific PCR could also facilitate the primary classification of unknown virus isolates. ***J. Med. Virol. 58:87–92, 1999.***

KEY WORDS: subgenus-specific primers; clinical applications; gastroenteritis; epidemic keratoconjunctivitis

INTRODUCTION

Human adenoviruses (Ad) are implicated in numerous diseases and are widespread throughout the world. Based on a variety of criteria such as neutralisation (SN), haemagglutination (HA), and DNA genome homologies, Ad have been divided into the six different subgenera A to F. Whereas subgenus F adenoviruses are considered to be second only to rotaviruses as a cause of infantile viral gastroenteritis [Uhnoo et al., 1984; Brandt et al., 1985; Kidd et al., 1986], subgenus A adenoviruses are isolated most commonly from infants with gastroenteritis in small outbreaks [Hammond et al., 1985; Adrian et al., 1987]. Respiratory diseases in small children are often caused by subgenus C adenoviruses, which show prolonged excretion after infection [Fox et al., 1977]. Furthermore, subgenus C adenoviruses seem to be associated with sudden infant death syndrome (SIDS) [Bajanowski et al., 1996]. Epidemic keratoconjunctivitis (EKC), a serious acute eye infection, is caused by subgenus DI adenoviruses [Jawetz et al., 1955; Guyer et al., 1975; de Jong et al., 1983]. Subgenus B adenoviruses are associated with acute respiratory infection, follicular conjunctivitis (cluster B:1), and haemorrhagic cystitis (cluster B:2) [Wadell, 1990]. Ad4, the only member of subgenus E, can also cause acute respiratory disease and conjunctivitis [Bennett et al., 1957; Murray et al., 1957].

Adenoviruses have been detected by polymerase chain reaction (PCR) in clinical samples of different origins [Allard et al., 1990; Kinchington et al., 1994; Jackson et al., 1996]. We also suggested previously applying PCR for the detection and typing of adenoviruses in clinical samples [Pring-Åkerblom and Adrian, 1994; Pring-Åkerblom et al., 1997]. Under clinical conditions, subgenus-specific identification is often sufficient, and it is less time consuming than serotype iden-

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tification. We therefore decided to extend the genus- and type-specific PCR by a subgenus-specific PCR.

The hexon is the major adenovirus capsid protein. It consists of three identical polypeptides, which exhibit a pseudohexagonal base rich in β-structure and a triangular top formed of the three surface loops l_1, l_2, and l_4 [Roberts et al., 1986; Athapilly et al., 1994]. Sequence analysis of several hexon genes [Akusjärvi et al., 1984; Kinloch et al., 1984; Toogood and Hay, 1988; Toogood et al., 1989; Pring-Åkerblom et al., 1995a] suggested that the loop l_4 region carries a subgenus-specific determinant. To confirm this observation, we analysed the corresponding hexon gene regions of further 10 serotypes belonging to different subgenera. Based on the hexon sequence data, we developed a multiplex, nonnested subgenus-specific PCR. Because the PCR primers generate amplimers of well-distinguishable length, there is no need for subsequent restriction enzyme analysis to differentiate between the subgenera. The applicability of this PCR for diagnostic purposes was tested with 100 clinical specimens of different origin.

MATERIALS AND METHODS

Viruses and Viral DNA

Viral prototypes (D1), subgenus A: Ad12 (Huie), Ad18 (DC), Ad31 (1315/63); subgenus B1: Ad3 (GB), Ad7 (Gomen), Ad16 (Ch79); subgenus B2: Ad11 (Slobitski), Ad14 (DeWitt), Ad34 (Compton), Ad35 (Holden); subgenus C: Ad1 (Ad71), Ad2 (Ad6), Ad5 (Ad75), Ad6 (Ton 99); subgenus DI: Ad8 (Trim), Ad9 (Hicks), Ad19 (587), Ad37 (GW); subgenus DII: Ad17 (Ch22); subgenus DIII: Ad28 (BP-5); subgenus E: Ad4 (RI-67); subgenus F: Ad40 (Dugan/Hovi), Ad41 (Tak); viral intermediate strains, subgenus B: Ad34/H11 (1545), Ad21/H21+35 (1333), Ad11+35/H11 (1334); subgenus D: Ad15/H9 (2003), Ad37/H17 (1582), Ad30/H44 (1468), Ad44/H13 (1587), Ad45/H44 (1308), 20′/Hx (1519) [Nötzel et al., 1985; Hierholzer et al., 1988]; subgenus C genome types, *Ad1:* D11, D12, D19, D21; *Ad2:* D24, D25, D27, D35; *Ad5:* D4, D10, D12, D17; *Ad6:* D2–D5 [Adrian and Wolf, 1989; Adrian et al., 1990]. The viruses were passaged several times in HeLa cells. Viral DNA for PCR was extracted from infected cells following as described previously [Doerfler, 1969].

Construction of Plasmids and DNA Sequencing

The loop l_4 region and parts of the flanking conserved hexon gene regions of Ad1, 6, 8, 9, 17, 18, 19, 28, and 37 were amplified using the primer pair $Hl_4$1/$Hl_4$2 ($Hl_4$1, forward primer: 5′-TGGAACTTCCGCAAGGA-3′; $Hl_4$2, reverse primer: 5′-GGAGAAGGGCGTGCGCA-3′), and the corresponding Ad31 region was amplified with $Hl_4$3 (forward primer: 5′-AGAGCACCCTGGGCAAT-3′) and $Hl_4$4 (reverse primer: 5′-TTAGGTGGTAGCGTTAC-3′). PCR was performed as described previously [Pring-Åkerblom and Adrian, 1995]. PCR products were cloned into pUC18. Both strands of several suitable clones were sequenced with internal primers. DNA sequence comparison was accomplished using DNASIS (Pharmacia). The sequences have been made generally available in the EMBL Database under Accession Nos. Y17244 (Ad1), Y17245 (Ad6), Y17246 (Ad8), Y17247 (Ad9), Y17248 (Ad17), Y17249 (Ad18), Y17250 (Ad19), Y17251 (Ad28), Y17252 (Ad37), and Y17253 (Ad31); a detailed annotation of the nucleotide sequences will not be shown.

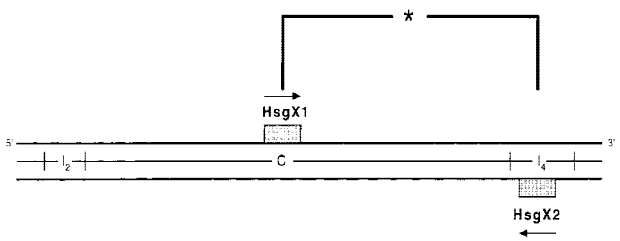

Fig. 1. Location of the subgenus-specific primers in the adenovirus hexon gene. HsgX1, primers HsgA1-HsgF1; HsgX2, primers HsgA2-HsgF2; *PCR amplimers of varying length (269p–586bp); l_2, loop l_2 hexon region; C, conserved hexon region; l_4, loop l_4 hexon region.

Specimens

The clinical specimens (65 stool samples, 23 ocular swabs, 12 throat swabs) were obtained from several University hospitals. The stool specimens were derived from patients with enteritis or gastroenteritis; the ocular swabs were obtained from patients with (beginning) signs of EKC; and the throat swabs from immunocompromised patients. Sample preparation and DNA purification were carried out following the QIAamp Blood Kit protocol (QIAGEN GmbH, Hilden).

Subgenus-Specific PCR Primers and PCR Procedure

The six subgenus-specific primer pairs HsgA1/HsgA2, HsgB1/HsgB2, HsgC1/HsgC2, HsgD1/HsgD2, HsgE1/HsgE2, and HsgF1/HsgF2 were evaluated after sequence comparisons. In addition to published sequences [Akusjärvi et al., 1984; Kinloch et al., 1984; Toogood and Hay, 1988; Toogood et al., 1989; Sprengel et al., 1994; Pring-Åkerblom et al., 1995a, 1995b], the sequences of the 10 above-listed serotypes (Ad1–Ad37) were included in the comparison.

The primers were designated "H" for hexon, "sg" for subgenus, and "A" to "F" for the corresponding subgenus. The HsgA1–F1 primers, which determine the length of the PCR products, target to the fairly conserved hexon region located between the hypervariable loop l_2 region and the variable loop l_4 region (Fig. 1). The HsgA2–F2 primers target to the loop l_4 region of the hexon gene. The subgenus-specific primer pairs are presented in Table I.

The primer pairs were tested for subgenus-specificity in PCR, using DNA of serotypes belonging to the corresponding subgenera. HsgA1/A2 was tested with Ad12, 18, and 31 DNA; HsgB1/B2 with Ad3, 7, 11, 14, 16, 34, 35, 34/H11, 21/H21+35, and 11+35/H11 DNA; HsgC1/C2 with Ad1, 2, 5, 6, *Ad1* D11, D12, D19, D21, *Ad2* D24, D25, D27, D35, *Ad5* D4, D10, D12, D17, and *Ad6* D2-D5 DNA; HsgD1/D2 with Ad8, 9, 17, 28, 19, 37,

TABLE I. Subgenus-Specific Primers for the Amplification of Adenoviruses

Subgenus	Primer		Amplimer length (bp)	
A	HsgA1	5′-AAGGTGTCAATYATGTTTG-3′		
A	HsgA2	5′-ACGGTTACTTKTTT-3′	HsgA1/A2	299
B	HsgB1	5′-TCTATTCCCTACCTGGAT-3′		
B	HsgB2	5′-ACTCTTAACGGCAGTAG-3′	HsgB1/B2	465
C	HsgC1	5′-ACCTTTGACTCTTCTGT-3′		
C	HsgC2	5′-TCCTTGTATTTAGTATC-3′	HsgC1/C2	269
D	HsgD1	5′-CCATCATGTTCGACTCCT-3′		
D	HsgD2	5′-AGGTAGCCGGTGAAGCC-3′	HsgD1/D2	331
E	HsgE1	5′-GACTCTTCCGTCAGCTGG-3′		
E	HsgE2	5′-GCTGGTAACGGCGCTCT-3′	HsgE1/E2	399
F	HsgF1	5′-ATTTCTATTCCTTCGCG-3′		
F	HsgF2	5′-TCAGGCTTGGTACGGCC-3′	HsgF1/F2	586

HsgA1–HsgF1, forward primers; HsgA2–HsgF2, reverse primers.

15/H9, 30/H44, 37/H17, 44/H13, 45/H44, and 20′/Hx DNA; HsgE1/E2 with Ad4 DNA; and HsgF1/F2 with Ad40 and Ad41 DNA. Cross-reactivity of the individual primer pairs were tested with DNA from serotypes belonging to the five remaining subgenera, i.e., in addition to Ad40 and Ad41, HsgF1/F2 was also tested with all the other under "viruses and viral DNA" listed serotypes belonging to the subgenera A–E.

For amplification of viral DNA obtained from cell culture, a 50-μl reaction mixture contained 10 ng of viral DNA, each primer at 0.5 μM, 5 μl QIAGEN PCR buffer (containing 1.5 mM $MgCl_2$), each dNTP at 200 μM, and 1 unit of QIAGEN HotstarTaq DNA Polymerase. Thermal cycling was performed for a total of 40 cycles. A cycle consisted of denaturation for 40 sec at 91°C, annealing for 30 sec at 40°C, and primer extension for 40 sec at 72°C. In the first cycle, the denaturation step continued for 900 sec at 95°C; in the last cycle, the extension step continued for 400 sec at 72°C. Five microliters of the final reaction products were analysed in a 2% agarose gel stained with ethidium bromide.

Multiplex PCR Procedure

The primers were then tested by multiplex PCR. In the multiplex PCR procedure, the reaction mixture contained not just the primers for one subgenus, but a master mix of all six subgenus-specific primer pairs (each primer at 0.5 μM). The multiplex PCR was carried out not only with DNA of individual serotypes, but also with DNA combinations (the viral DNA each at 10 ng) of up to six serotypes, each from a different subgenus. The clinical specimens were also tested by the multiplex PCR procedure. For amplification of viral DNA extracted from clinical samples, 30 μl of the purified DNA was used. Thermal cycling was performed as described above. Ten microliters of the final reaction products were analysed in a 2% agarose gel stained with ethidium bromide.

To establish the sensitivity of the multiplex PCR, amplification of known amounts of DNA samples were undertaken. The DNA suspensions were diluted to obtain a theoretical range of virus particles from 10^6 to 1 per reaction mixture (0.384 fg of Ad DNA corresponds to a single copy of linear double-stranded DNA of about 35,000 bp).

RESULTS

Sequence Analysis of the Hexon Loop l_4

In order to evaluate subgenus-specific PCR primers, we cloned and sequenced the hexon loop l_4 gene regions of Ad1, 6, 8, 9, 17, 18, 19, 28, 31, and 37. The analysed loop l_4 hexon regions were conserved within a given subgenus, with nucleotide homologies ranging between 78.4% (90.4% protein level) for subgenus A serotypes Ad18 and Ad31, and 99.3% (100% protein level) for subgenus B serotypes Ad3 and Ad7. Between serotypes of different subgenera, the loop l_4 was more variable, e.g., for Ad2/Ad3 a DNA homology of 65.3% (69.2% protein level), for Ad2/Ad40 a DNA homology of 69.7% (69.2% protein level), and for Ad3/Ad31 a DNA homology of 68.5% (71.2% protein level) was observed. Although the loop l_4 region of subgenus A is less conserved than the corresponding region of the other subgenera, which could be expected as the serotypes of this subgenus show the least overall sequence homology, it was possible to evaluate a subgenus A-specific PCR primer pair.

Primer Specificity

The specificity of the six evaluated primer pairs were tested on 23 adenovirus prototypes, representing all six subgenera, on 9 intermediate strains from subgenera B and D, and on 16 subgenus C genome types. For subgenus A, C, E, and F, all existing prototypes were tested with the corresponding subgenus-specific primer pair (subgenus A: HsgA1/A2, subgenus C: HsgC1/C2, subgenus E: HsgE1/E2, subgenus F: HsgF1/F2). For subgenus B (HsgB1/B2) and D (HsgD1/D2), several prototypes were selected, including the causative agents of pharyngoconjunctival fever, haemorrhagic cystitis, and EKC. Positive results were obtained with all prototypes when analysed on a 2% agarose gel. Serologically intermediate strains may be defined as strains that are related to one (or two) type(s) in SN and to another type(s) in haemagglutination inhibition tests (HI). They are a regular feature of subgenera B and D. To make sure that intermediate strains are also

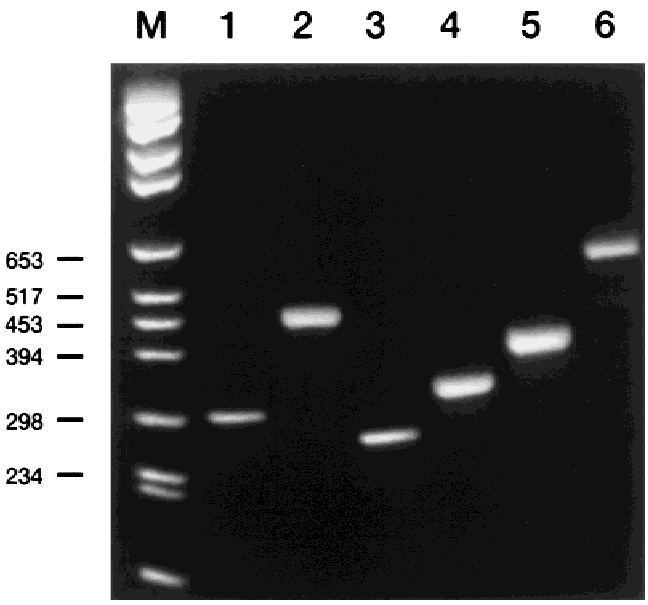

Fig. 2. Agarose gel electrophoresis of subgenus-specific PCR products. M, DNA molecular weight standard VI (Boehringer, Mannheim); Lane 1, subgenus A amplimer (HsgA1/A2); lane 2, subgenus B amplimer (HsgB1/B2); lane 3, subgenus C amplimer (HsgC1/C2); lane 4, subgenus D amplimer (HsgD1/D2); lane 5, subgenus E amplimer (HsgE1/E2); lane 6, subgenus F amplimer (HsgF1/F2).

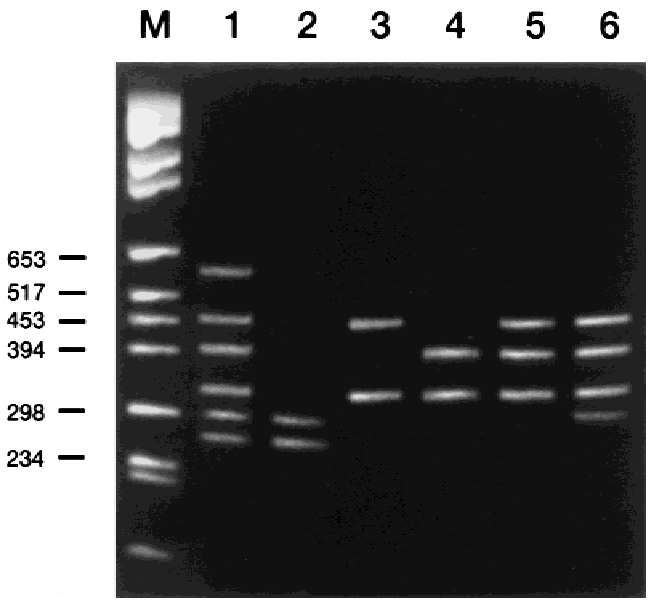

Fig. 3. Agarose gel electrophoresis of multiplex, subgenus-specific PCR products. M, DNA molecular weight standard VI (Boehringer, Mannheim). Lane 1, subgenus A–F amplimers; lane 2, subgenus A and C amplimers; lane 3, subgenus B and D amplimers; lane 4, subgenus E and D amplimers; lane 5, subgenus B, E, and D amplimers; lane 6, subgenus B, E, D, and A amplimers. (Beginning with lane 2, the PCR products are listed in order of decreasing amplimer length.)

amplified by subgenus-specific PCR, 3 subgenus B and 6 subgenus D intermediate strains were tested. PCR was also carried out with 16 subgenus C genome types, using the subgenus-specific primer pair HsgC1/C2. All intermediate strains and genome types showed positive results in subgenus-specific PCR. In Figure 2, the six subgenus-specific PCR products are shown. The length of the amplimers range between 269 bp for Subgenus C and 586 for subgenus F. Although the subgenus A-specific primers HsgA1 and A2 each contain a wobble base, no cross-reaction was observed with serotypes of other subgenera. Also for the other five primer pairs, no cross-reactivity was detected.

Multiplex, Subgenus-Specific PCR

Because it is desirable to have a one-step assay that detects adenoviruses of all subgenera, making it possible to distinguish easily between different subgenera without subsequent restriction analysis, and that can also detect serotypes of different subgenera in clinical samples, we established a multiplex, subgenus-specific PCR. The multiplex PCR assay contains all six subgenus-specific primer pairs. The six subgenus-specific PCR products generate a DNA ladder (Fig. 3), making it easy to distinguish between the different subgenera. As in the single-primer pair subgenus-specific PCR, no cross-reactivity was observed in the multiplex PCR. When adding the DNA of only one serotype, also only a single PCR band (corresponding in length to the given subgenus) could be detected. When several DNA samples were added, the multiplex PCR could amplify simultaneously up to four serotypes of different subgenera. Corresponding PCR products are shown in Figure 3. The PCR product ladder (Fig. 3, lane 2) was generated by mixing all six subgenus-specific PCR products prior to being loaded onto the agarose gel. This DNA ladder could be used instead of a commercial DNA molecular weight standard. The multiplex PCR assay was able to detect 10^2 virus particles.

Multiplex, Subgenus-Specific PCR Analysis of Clinical Specimens

Multiplex, subgenus-specific PCR was also conducted directly with 100 specimens of different origin. As shown in Table II, adenoviruses of all subgenera could be detected. Altogether 41 samples were adenovirus positive, thereof 26 stool samples, 13 ocular swabs, and 2 throat swabs. Although subgenus B adenoviruses were detected in all three different specimens, the other subgenera were restricted to specific specimens. Subgenus A, C, and F adenoviruses were only found in stool samples, subgenus D and E were only found in ocular swabs. One stool sample was not only positive for subgenus C but also for subgenus F adenoviruses. In Figure 4, multiplex, subgenus-specific PCR products of clinical specimens are shown (the double-positive PCR can be seen in lane 4). The samples were also tested by genus- and/or type-specific PCR, with consistent results (not shown).

DISCUSSION

We identified a subgenus-specific region in the adenovirus hexon loop l_4. By using primer pairs targeted to this variable hexon region and a conserved hexon

TABLE II. Multiplex, Subgenus-Specific PCR Analysis of Clinical Samples

Specimens derived from	No. of specimens	PCR positive	Subgenus A	Subgenus B	Subgenus C	Subgenus D	Subgenus E	Subgenus F
Stool	65	26[a]	4	2	7			14
Eye	23	13		1		10	2	
Throat	12	2		2				

PCR, polymerase chain reaction.
[a]One stool sample was positive for subgenus C and F.

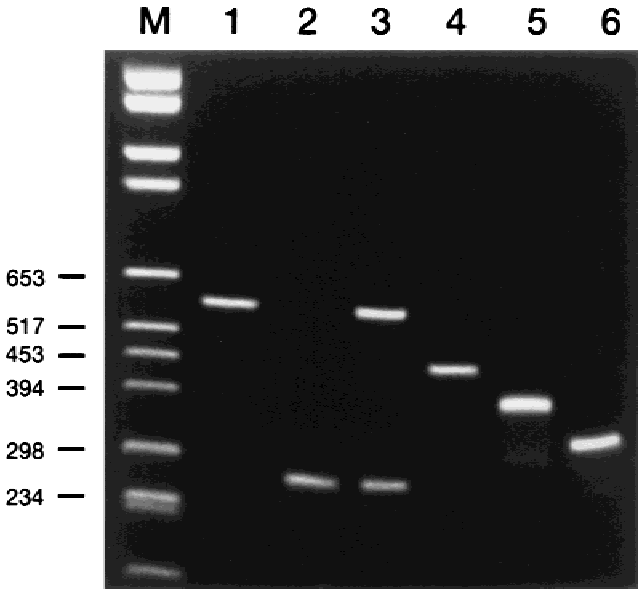

Fig. 4. Agarose gel electrophoresis of multiplex, subgenus-specific PCR products amplified directly from clinical specimens. M, DNA molecular weight standard VI (Boehringer, Mannheim); Lane 1, subgenus F amplimer (stool sample); lane 2, subgenus C amplimer (stool sample); lane 3, subgenus F and C amplimers (stool sample); lane 4, subgenus B amplimer (throat swab); lane 5, subgenus E amplimer (ocular swab); lane 6, subgenus D amplimer (ocular swab).

region, a multiplex, nonnested PCR for the detection and subgenus-specific identification of adenoviruses could be established. Because the six subgenus-specific amplimers are well distinguishable in agarose gel electrophoresis, subsequent restriction analysis is not necessary. This method is not only of considerable advantage because time is saved, but also because DNA cleavage of PCR products can be incomplete, leading to indistinct or even false results. Furthermore, the application of restriction enzyme digestion can be limited if the band intensity of PCR products is weak (a problem especially occurring with clinical specimens) or if background bands are visible. Kidd et al. [1996] presented a subgenus-specific identification of adenoviruses by PCR that requires a subsequent restriction analysis of subgenus B:1, C, D, and E PCR products for a reliable differentiation. The advantage of this PCR assay is that only three primers are used. But although we used all six subgenus-specific primer pairs in the multiplex PCR, decreasing the number of primers is also possible by using only a combination of primers related to disease and/or clinical specimen. In clinical application, e.g., a combination of subgenus B, D, and E primers would mostly be sufficient for ocular swabs from patients with eye infections, a combination of subgenus A, B, C, and F primers for stool samples from patients with gastroenteritis and/or respiratory diseases, and the single subgenus B primer pair for urine samples from patients with haemorrhagic cystitis.

In comparison with the genus-specific PCR, the subgenus-specific PCR has several advantages. Especially the rapid, one-step differentiation between subgenus D adenoviruses, causing the severe and highly contagious EKC, and subgenus B and E adenoviruses, causing relative harmless ocular infections, is of great importance. So far there are no effective antiadenoviral drugs, and immediate prophylaxis, including appropriate personal hygiene, is the most important means for preventing the spreading of EKC during acute epidemics. Unfortunately, the subgenus-specific PCR cannot distinguish between the EKC-causing adenoviruses and other subgenus D adenoviruses such as Ad9, Ad15/H9, or Ad17, which cause a relatively harmless conjunctivitis. For epidemiological studies, the serotype could be determined by a subsequent type-specific PCR with Ad8, Ad19, and Ad37 primers [Pring-Åkerblom et al., 1997], and/or conventional SN (the application of HI is limited here, because Ad8, Ad9, and Ad15/H9 show cross-reaction). Differentiation between genome types can only be accomplished by time-consuming DNA restriction analysis.

As the subgenus C adenoviruses show a high genetic variability within a given serotype that eminently exceeds that of other serotypes of subgenera A, B, D, and E [Adrian and Wolf, 1989; Adrian et al., 1990], PCR was also performed with 16 subgenus C genome types. Because the DNA of all genome types could be amplified, a conserved l_4 hexon region within a given subgenus can be proposed. This possibility is important for a general application of the subgenus-specific PCR, especially as genome types often show a distinct geographic distribution. Furthermore, we amplified the DNA of subgenus B and D intermediate strains successfully. Intermediate strains arise by homologous recombination of two serologically different parent viruses [Boursnell and Mautner, 1981]. Because both parental strains belong to the same subgenus, the subgenus can be identified by PCR, whereas further serotype classification must involve SN and HI. Besides intermediate strains, new subgenus B and (foremost) subgenus D serotypes emerge in the human population. Recently, new serotypes were isolated from the stool and urine of AIDS patients [de Jong et al., 1996]. Because several

already known adenoviruses show no HA or an atypical HA pattern [Wigand, 1970; Mei and Wadell, 1993], subgenus-specific PCR could facilitate the primary classification of unknown virus isolates into subgenera.

It was demonstrated that the multiplex PCR could amplify simultaneously the DNA of four distinct subgenera. However, this multiple amplification might not be necessary for the normal application of the subgenus-specific PCR. Nevertheless, there was one stool sample that contained subgenus C and subgenus F adenoviruses. The patient could have been infected acutely with both viruses, possibly leading to a more severe course of disease, or another likely explanation could be that the subgenus C viruses showed prolonged excretion after a previous infection.

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